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Identification and *In Vitro* Characterization of a Marek's Disease Virus–Encoded Ribonucleotide Reductase

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SUMMARY. Marek's disease virus (MDV) encodes a ribonucleotide reductase (RR), a key regulatory enzyme in the DNA synthesis pathway. The gene coding for the RR of MDV is located in the unique long (UL) region of the genome. The large subunit is encoded by UL39 (RR1) and is predicted to comprise 860 amino acids whereas the small subunit encoded by UL40 (RR2) is predicted to be 343 amino acids long. Immunoprecipitation analysis of MDV-1 (GA strain)-infected cells with T81, a monoclonal antibody specific for RR of MDV, identified two major proteins of 90,000 and 40,000 daltons, corresponding to RR1 and RR2, respectively. In addition, RR was abundantly expressed in the cytoplasm of cells infected with 51 strains of MDV belonging to MDV serotypes 1, 2, and 3 as demonstrated by immunofluorescence staining. Northern blot analysis of RNA extracted from MDV-infected cells showed a major band of around 4.4 kb in size corresponding to the RR1 and RR2 transcripts. *In vivo*, RR was abundantly expressed in lymphoid organs and feather follicle epithelium of MDV-infected chickens during early cytolytic infection, as determined by immunohistochemistry. There was, however, no expression of RR in MDV-induced tumors in lymphoid organs. The abundant expression of RR in MDV-infected chicken may suggest an important role of RR in the conversion of ribonucleotides to deoxyribonucleotides for MDV DNA synthesis.

RESUMEN. Identificación y caracterización *in vitro* de la ribonucleótido reductasa codificada por el virus de Marek.

El virus de la enfermedad de Marek (MDV) codifica una enzima ribonucleótido reductasa (RR), que es una enzima reguladora clave en la ruta de la síntesis de ADN. El gene que codifica para la ribonucleótido reductasa del virus de Marek se encuentra en la región única larga (UL) del genoma. La subunidad grande está codificada por UL39 (RR1) y se predice que comprende 860 aminoácidos mientras que la subunidad pequeña codificada por UL40 (RR2) que se prevé que tiene 343 aminoácidos de longitud. El análisis por inmunoprecipitación de células infectadas por el virus de Marek serotipo 1 (cepa GA) utilizando el anticuerpo monoclonal T81, que es específico para la ribonucleótido reductasa del virus de Marek, identificó dos proteínas principales de 90,000 y 40,000 daltons, que corresponden a las RR1 y RR2, respectivamente. Además, la ribonucleótido reductasa fue expresada abundantemente en el citoplasma de las células infectadas con 51 cepas del virus de Marek pertenecientes a los serotipos de MDV 1, 2 y 3 como se demostró por la tinción de inmunofluorescencia. El análisis de transferencia Northern con ARN extraído de células infectadas con el virus de Marek mostraron una banda principal de alrededor de 4.4 Kb de tamaño correspondientes a los transcriptos de RR1 y RR2. La ribonucleótido reductasa fue expresada abundantemente *in vivo* en órganos linfoides y en el epitelio folículo de la pluma de pollos infectados con el virus de Marek durante la infección citolítica temprana, tal como se determinó por inmunohistoquímica. Sin embargo, no hubo expresión de la ribonucleótido reductasa en tumores de órganos linfoides inducida por el virus de Marek. La expresión abundante de la ribonucleótido reductasa en pollos infectados con el virus de Marek puede sugerir un papel importante de esta enzima en la conversión de ribonucleótidos a desoxirribonucleótidos para la síntesis de ADN de este virus.

Key words: chicken, Marek's disease, Marek's disease virus, riboncleotide reductase

Abbreviations: CEF = chicken embryo fibroblasts; DEF = duck embryo fibroblasts; EBV = Epstein Barr virus; FFE = feather follicle epithelium; HSV = herpes simplex virus; HVT = turkey herpesvirus; IFA = immunofluorescence assay; IHC = immunohistochemistry; Mab = monoclonal antibody; MD = Marek's disease; MDV = Marek's disease virus; PolyA = polyadenylation; rMd5 = recombinant Md5 virus; RR = ribonucleotide reductase; RR1 = large subunit of RR; RR2 = small subunit of RR; SDS-PAGE = sodium dodecyl sulfate–polyacrylamide gel electrophoresis; UL = unique long region of MDV genome

Marek's disease virus (MDV) is a member of the genus *Mardivirus,* subfamily *Alphaherpesvirinae* in the family *Herpesviridae* (27). MDVs are classified into three closely related but distinct groups. Serotype 1 viruses (Gallid herpesvirus 2) cause an acute lymphoproliferative disease, namely Marek's disease (MD) in chickens, resulting in T cell lymphomas that metastasize to visceral organs and peripheral nerves (5,19). Serotypes 2 (Gallid herpesvirus 3) (26) and 3 viruses (Meleagridis herpesvirus 1) (35) are nonpathogenic and were isolated from chickens and turkeys, respectively.

MD can be prevented by vaccination with all three serotypes of MDV (21,33,34) which share similar genomic organization, significant DNA homology, and antigenic cross-reactivity (3). Despite the widespread use of vaccination in chickens, MD is still a major problem for the poultry industry around the world requiring a suitable strategy for prevention of the disease (32). We have been involved in the study of MDV gene function and construction of recombinant vaccines by developing mutant viruses with deletions of genes contributing to MDV virulence.

The complete DNA sequence from the three serotypes has made it possible to identify many previously unknown genes and open reading frames (1,14,17,30). Viral proteins or antigens common to three serotypes of MDV have stimulated considerable interest,

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mainly because they might be responsible for the protection afforded by vaccine strains. In an attempt to identify genes that encode common antigens involved in vaccine protection, a monoclonal antibody (Mab T81) specific for a common MDV antigen was used to screen a genomic expression library and identified a recombinant MDV phage clone, GB. Sequence analysis of the GB clone revealed an open reading frame encoding a protein homologous to the HSV large subunit of a ribonucleotide reductase (RR) (9).

RR is an essential enzyme for the conversion of ribonucleotides to deoxyribonucleotides in prokaryotic and eukaryotic cells (23). RR has been divided into three classes according to its mechanism for generation of the protein radical, metal cofactor requirement, and subunit composition (15). RR from mammalian cells, like most eukaryotic cells, belongs to class 1a, similar to the well-characterized RR of *Escherichia coli*. Class 1a has an $\alpha_2\beta_2$ structure consisting of two homodimeric subunits, proteins R1 (α_2) and R2 (β_2). The R1 protein contains an active site and a binding site for an allosteric effector. The R2 protein has a radical storage device containing an iron center–generated tyrosyl free radical (15).

Alphaherpesviruses express a functional RR required for virus growth in nondividing cells and for viral pathogenesis and reactivation from latency in infected host cells (7,8). The RR of HSV-1 is the most extensively characterized and, like the mammalian and *E. coli* enzymes, belongs to class 1a but completely lacks allosteric regulation (8). Several herpesviruses, namely HSV-1 and HSV-2, Epstein Barr virus (EBV), pseudorabies virus, and equine herpesviruses, induce a RR activity with properties different from those of the cellular enzyme. The herpes simplex virus (HSV) enzyme is composed of two nonidentical subunits (RR1 and RR2) that associate to form an active holoenzyme. The amino terminus of HSV-1 RR1 encodes a protein kinase that is absent in other herpesviruses but present in HSV-2. The amino acid sequence of the protein kinase region between HSV-1 and HSV-2 shares only 38% similarity (20,22).

In herpesviruses, the RR genes are not essential for virus growth in tissue culture and thus are good candidates for deletion mutants (4,13). An RR mutant of HSV showed reduced virulence and altered latency and reactivation characteristics in the mouse model (4). RR mutants of pseudorabies virus are avirulent for pigs, and provide partial protective immunity against virulent challenge (10).

In this report, we compare the RR protein sequence of MDV to that of other herpesviruses. In addition, we examined the expression of RR RNA by northern blot analysis and the expression of RR protein in cell culture and *in vivo* by immunoprecipitation, immunofluorescence, and immunohistochemistry. Our results indicate that RR is highly expressed *in vitro* and *in vivo*, suggesting it plays an important role in the biology of MDV.

MATERIALS AND METHODS

Cells and Viruses. Three MDV serotypes were used in this study. Serotype 1 viruses included GA/22, virulent pathotype (11); Md5, very virulent pathotype (32); 648A, very virulent plus pathotype (32); and CVI988/Rispens (24). One serotype 2 virus was used: SB-1 (26), as well as one serotype 3 virus: turkey herpesvirus (HVT) (35). All other viruses used in this study are listed in Table 2. Chick embryo fibroblast (CEF) and duck embryo fibroblast (DEF) cultures were grown at 37 C in Liebowitz-McCoy medium supplemented with 4% calf serum. Confluent cultures were infected with cell-associated MDV at a multiplicity of infection of approximately 0.3 and maintained in the same medium with 1% calf serum. When the cultures displayed extensive cytopathic effect, they were harvested for extraction of both DNA and RNA.

Chickens. Chickens used in this study were from the $15I_5 \times 7_1$ hybrid chicks (2) free of maternal antibodies to all three serotypes of MDV. The breeder chickens were maintained at the Avian Disease and Oncology Laboratory (East Lansing, MI) and were free of antibodies to avian leukosis virus, reticuloendotheliosis virus, and various other poultry pathogens.

Northern blot analysis. Total RNA was isolated from mock-infected DEF cells and DEF cells infected with the GA strain of MDV using the guanidinium-phenol:chloroform method as described previously (25). Twenty micrograms of RNA were loaded into each well of the 96-well plate, electrophoresed through 1.2% formaldehyde gel, and transferred onto a Hybond-nylon membrane as described previously (25). A 2.5-kb fragment containing the entire RR gene was used as probe and labeled using a ^{32}P-dCTP (NEN Life Sciences, Inc., Boston, MA). Northern blot hybridization was performed using a standard procedure (25). Transcript size was determined by comparison to the RNA ladder marker (New England Biolabs, Inc., Ipswich, MA).

Immunoprecipitation. DEF infected with GA strain (serotype 1), SB-1 (serotype 2), and HVT (serotype 3) were metabolically labeled with 50 µci/ml of ^{35}S-methionine (NEN Life Sciences). After 5 hr, the radioactive medium was removed, monolayers were washed twice with phosphate-buffered saline, and cell lysates were prepared with lysis buffer. Lysates were precleaned by incubation with normal mouse ascites fluids, and protein A-Sepharose CL-4B (Amersham Biosciences, Piscataway, NJ). Precleaned supernatants were then subjected to further immunoprecipitation with T81, a monocloned antibody specific for UL-39. Samples were boiled for 5 min in 2× Laemmli buffer with 2-mercaptoethanol and analyzed by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (28). The gels were fixed, treated with 1.0 M salicylate, and dried prior to exposure to a Kodak XAR-5 film.

Indirect immunofluorescence assay (IFA) and immunohistochemistry (IHC). IFA of MDV-infected DEF grown on 35-mm dishes was carried out as previously described (16). For IHC, lymphoid organs (thymus, spleen, bursa of Fabricius), skin with feather follicles, and GA-induced tumors from infected and uninfected chickens were embedded in optimal-cutting-temperature compound (Sakura Finetek U. S. A., Inc., Torrance, CA), immediately frozen in liquid nitrogen, and stored at −80 C until used. Four- to eight-micrometer-thick cryostat sections of tissue were prepared, fixed with cold ethanol for 5 min, and air-dried. Immunostaining was carried out using the Vectastain ABC Kit (Vector Laboratories, Inc., Burlingame, CA), according to the manufacturer's instructions. For IFA staining, Mab T81 was used at a working dilution of 1:300. For IHC staining of lymphoid organs, skin, and tumors, Mab T81 was used at a working dilution of 1:2000; a dilution of 1:1000 was used for polyclonal antibodies specific for the oncoprotein Meq as positive control for tumor cells.

***In vivo* experiments.** One-day-old $15I_5 \times 7_1$ chicks from unvaccinated breeder hens free of antibodies to all three MDV serotypes were wing-banded at hatch, randomly sorted into two experimental groups, and held in modified Horsfall-Bauer isolators for the duration of the 8-wk experimental period. One group of 13 chickens served as a negative control while the other group of 22 chickens was inoculated intra-abdominally with 2000 plaque-forming units of very virulent recombinant Md5 (rMd5) virus. For identification of RR in MDV-infected chickens during early cytolytic infection, five chickens each from rMd5-infected and uninoculated control groups were euthanatized 6 days postinoculation; lymphoid organs (thymus, bursa of Fabricius, and spleen) were collected and examined for RR expression by IHC as described in the previous section. Skin tissues containing feather follicles from two rMd5-infected chickens were collected at 21 days.

RESULTS

Sequence analysis of MDV RR gene. The MDV RR gene is located in the unique long (UL) region of the genome. The RR1

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              -------------XXXXXHVVKRDGSXERXMFDKIASXXXKXXXG-XXHF   Majority
                       10        20        30        40        50
1             -------------MNQNLLVTKRDGSTERINLDKIHRVLDWAAEG--LHN   E coli RR1 ZP_02742225
1             -----------------MHVVKRDGRQERVMFDKITSRIQKLCYGLNADF   Gallus gallus RR1 NP_001026008
1             VPLELTPENAEAVARFLGDAVNREPALMLEYFCRCAREETKRVP--PRTF   HSV-1 RR1 truncated
1             --MDHSDVYEPYTFSGKHHVRMYDFSKLTPMWNKDASTPPRKFA--DMHI   MDV Rock RR1 protein

              VSPXQXTLXXIXX-LLNXXTLVEMETLXXDVAXXPXXXXPDYXXLAARTA   Majority
                       60        70        80        90       100
36            VSISQVELRSHIQFYDGIKTSDIHETIIKAAADLISRDAPDYQYLAARLA   E coli RR1 ZP_02742225
34            VDPAQITMKVIQG-LYSGVTTVELDTLAAETAATLTTKHPDYAILAARIA   Gallus gallus RR1 NP_001026008
49            GSPPRLTEDDFG--LLNY-ALVEMQRLCLDVPPVPPNAYMPYYLREYVTR   HSV-1 RR1 truncated
47            ASTVAMELYNIERELLNMGHLSGMETMMVDVRKSPEMIPSTINMRDHITR   MDV Rock RR1 protein

              LVN--KPLXKXXXXRLFSXXGXLVHLRIXXREGKXDXWLLSKXLXLXFXXX   Majority
                      110       120       130       140       150
86            IFH----LRKKAYGQFEPPALYDHVVKMVEMGKYDNHLLEDYTEEEFKQM   E coli RR1 ZP_02742225
83            VSN----LHKETKKVFSDVMDDLYNYINPHNGKHSPMISKETLDIVLANK   Gallus gallus RR1 NP_001026008
96            LVNGFKPLVSRSARLYRILGVLVHLRIRTREASFEEWLRSKEVALDFGLT   HSV-1 RR1 truncated
97            LVNKMKPICRFDERLYSLCGELVHLRIELREVPLDTWLLSKKLNLKYEVV   MDV Rock RR1 protein

              DRLRXHXAX--I----L--Y-----S----L---GLDSALKYEEFXVKXL   Majority
                      160       170       180       190       200
132           DTFIDH------------------------------DRDMTFSYAAVKQL   E coli RR1 ZP_02742225
129           DRLNSAIIY---------------------------DRDFSYNYFGFKTL   Gallus gallus RR1 NP_001026008
146           ERLREHEAQLVILAQALDHYDCLIHSTPHTLVERGLQSALKYEEFYLKRF   HSV-1 RR1 truncated
147           DNIRRYRAH--IEMDMLRFYG----SIHPWLKRLGLQSALKYEEYLVELE   MDV Rock RR1 protein
                                                  1

              EGK--ES-XQXXXXRIAGXXAERAQXX---MXXXAXGX--XXNWXXXFKXF   Majority
                      210       220       230       240       250
152           EGK-----YLVQNRVTGEIYESAQFLYILVAACLFSNYPRETRLQYVKHF   E coli RR1 ZP_02742225
152           ERS-------YLLKINSKVAERPQHM---LMRVAVGI--HKNDIDAAIET   Gallus gallus RR1 NP_001026008
196           GGHYMESVFQMYTRIAGFLACRATRG---MRHIALGR--EGSWWEMFKFF   HSV-1 RR1 truncated
191           DGK-KESLCQFFVRLAAAAATEASMKKPFMTTLTTG---VANWRTTFTTF   MDV Rock RR1 protein

              FXALSXXXIVPSTPXMLNLGTXXXXTSSCYLXXPXXDSXXXTLXAITSXV   Majority
                      260       270       280       290       300
197           YDAVSTFKISLPTPIMSGVRTPTRQFSSCVLIECG-DSLDS-INATSSAI   E coli RR1 ZP_02742225
190           YNLLSERWFTHASPTLFNAGTNRPRLSSCFLLCMKDDSIEG-IYDTLKQC   Gallus gallus RR1 NP_001026008
241           FHRLYDHQIVPSTPAMLNLGTRNYYTSSCYLVNPQATTNKATLRAITSNV   HSV-1 RR1 truncated
237           FLALANQLFVPSTPCMLFLGREGTSTASCYLMDPRTNNTQDTLKAITEDV   MDV Rock RR1 protein
                                         2

              VXILXXRGGIGLXVQXINAXGS-I-G-XXGXXGLMPXLKVLDSLVXAXNQ   Majority
                      310       320       330       340       350
245           VKYVSQRAGIGINAGRIRALGSPIRGGEAFHTGCIPFYKHFQTAVKSCSQ   E coli RR1 ZP_02742225
239           ALISKSAGGIGLAVSCIRATGSYIAGTNGNSNGLVPMLRVYNNTARYVDQ   Gallus gallus RR1 NP_001026008
291           SAILARNGGIGLCVQAFNDS-------GPGTASVMPALKVLDSLVAAHNK   HSV-1 RR1 truncated
287           VPHLLARGGIGISLQHLN-----------GKFGLMHVMKVLDSLVMAANV   MDV Rock RR1 protein
                    3

              G-XXRPGGACVYLEPWHLDVXSXLXLKGNXGXEE-QRCDNXFXALWIPDL   Majority
                      360       370       380       390       400
295           G-GVRGGAATLFYPMWHLEVESLLVLKNNRGVEG-NRVRHMDYGVQINKL   E coli RR1 ZP_02742225
289           GGNKRPGAFAIYLEPWHLDIFEFLDLKKNTGKEE-QRARDLFFALWIPDL   Gallus gallus RR1 NP_001026008
334           E-SARPTGACVYLEPWHTDVRAVLRMKGVLAGEEAQRCDNIFSALWMPDL   HSV-1 RR1 truncated
326           N-ESRPTGICVYLEPWHADIMSALNMRGMVAAEESRRCDNVFLALWSCDL   MDV Rock RR1 protein
                         4

              FFKRLLRHXDGEKNV-WTLFDPXXSPGLXDXHG---EEFEKLYEXLEKXG   Majority
                      410       420       430       440       450
343           MYTRLLKGED------ITLFSPSDVPGLYDAFFADQEEFERLYTKYEKDD   E coli RR1 ZP_02742225
338           FMKRVETNQD------WSLMCPNECPGLDEVWG---EEFEKLYESYEKQG   Gallus gallus RR1 NP_001026008
383           FFKRLIRHLDGEKNVTWTLFDRDTSMSLADFHG---EEFEKLYQHLEVMG   HSV-1 RR1 truncated
375           LFKRYLRHVNGEKNVIWTLFDSRASI-LTKLHS---EEFEKEYERLESEG   MDV Rock RR1 protein
                5                              6

              XGR-XXVKAQELXYAIIXSAASTGSPFILFKDACNRHSIXDTQGAAIAGS   Majority
                      460       470       480       490       500
387           SIRKQRVKAVELFSLMMQERASTGRIYIQNVDHCNTHSPFDPAIAPVRQS   E coli RR1 ZP_02742225
379           RVR-RVVKAQQLWYAIIESQTETGTPYMLYKDSCNRKSNQQNLG-TIKCS   Gallus gallus RR1 NP_001026008
430           FG--EQIPIQELAYGIVRSAATTGSPFVMFKDAVNRHYIYDTQGAAIAGS   HSV-1 RR1 truncated
421           LGV-ASIPIRDMMFAIIKSAASTGSPFILFKDACNRHYITNTQGDAIAGS   MDV Rock RR1 protein
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Fig. 1. Alignment of protein sequences of RR-1 between MDV, HSV, chicken and *E. coli*. The rectangles in the figure indicate blocks of similar amino acid sequences.

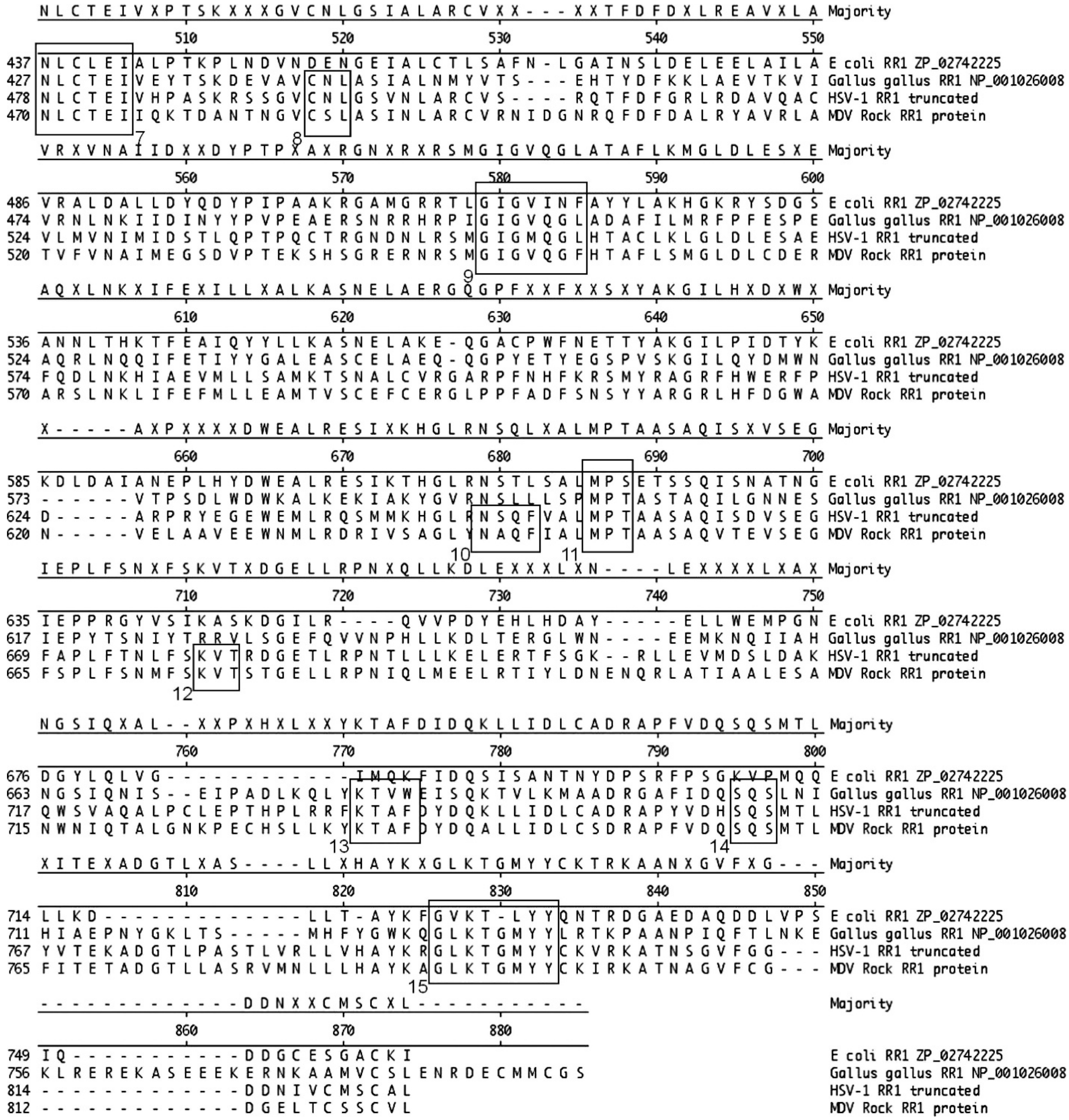


Fig. 1. Continued.

subunit, an 822-amino-acid–long protein with a calculated molecular mass of 92,899 daltons, is encoded by UL39 whereas the RR2 subunit, a 343-amino-acid–long protein with an estimated molecular mass of 39,100 daltons, is encoded by UL40. The deduced amino acid sequences of RR1 and RR2 from MDV serotype 1 were aligned and compared to that of HSV-1, chicken, and *E. coli* and are shown in Figs. 1 and 2. Twenty-one blocks of conserved amino acids were detected between the RR of MDV and that of HSV-1; 15 blocks between MDV and chicken; and eight blocks between MDV and *E. coli* (Table 1). Therefore, MDV RR shares higher similarity to HSV-1, second to chicken RR, and least to *E. coli*. As shown in Fig. 1, there are five conserved cysteine residues in RR1 located in blocks 2, 7, and 8, whereas the other two cysteine residues, amino acids 869 and 872, are at the carboxyl terminus. In block 9, there are three conserved glycine residues within the sequence G-I-G-V-Q-G, which is predicted to be the site for nucleotide binding (31).

In RR2, the histidine and tyrosine at amino acid positions 175 and 179, respectively, are the predicted locations of the iron-binding site and tyrosine free radical (Fig. 2). There is a nonapeptide,

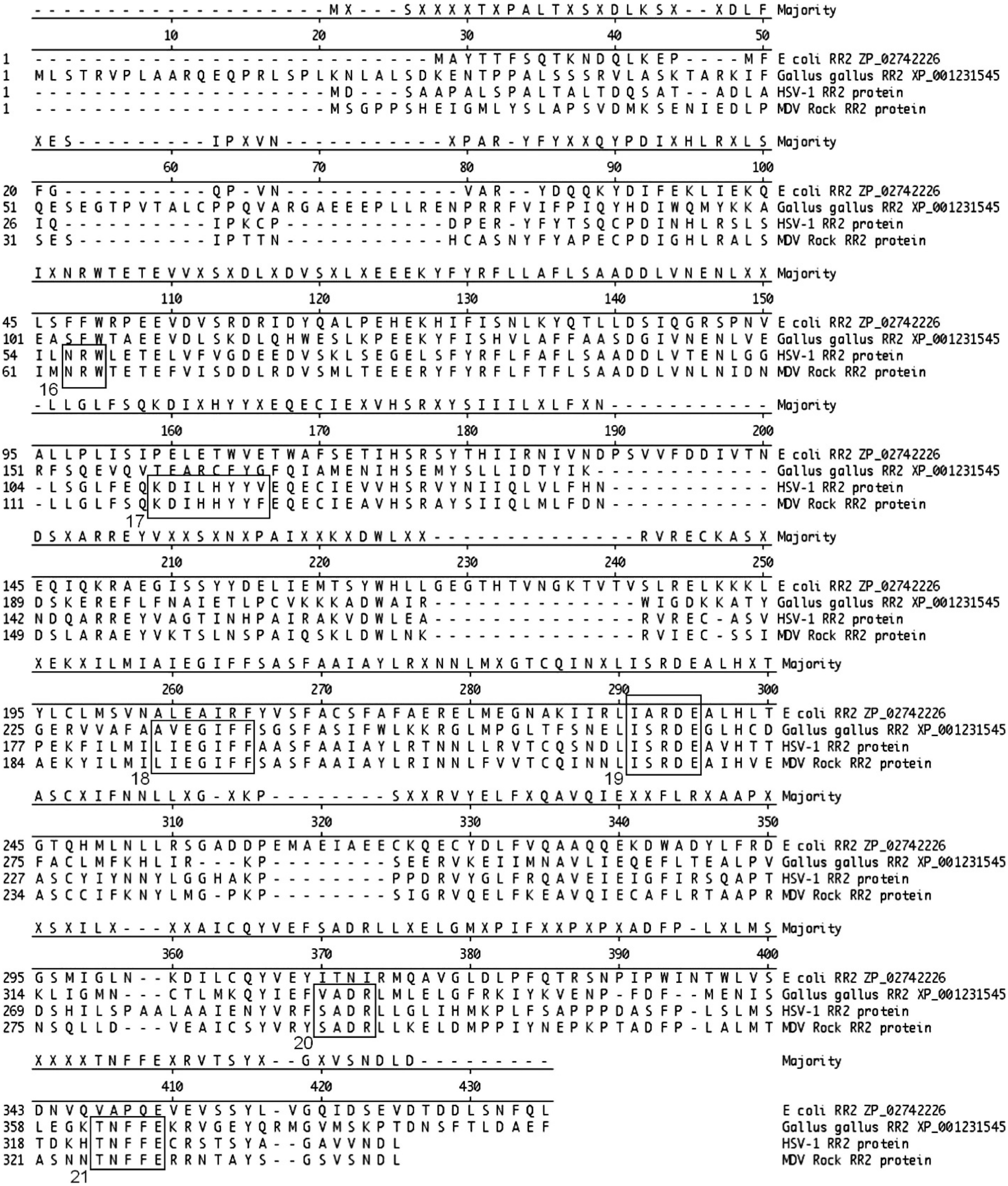


Fig. 2. Alignment of protein sequences of RR-2 between MDV, HSV, chicken and *E. coli*. The rectangles in the figure indicate blocks of similar amino acid sequences.

YAGAVVNDL, which corresponds to the non–carboxy-terminal amino acids of RR2 reported to specifically inhibit HSV RR activity (6). In MDV, the nonapeptide YSGSVSNDL is different from that of HSV.

Identification of RR protein in MDV-infected cells by immunofluorescence. Expression of RR in MDV-infected cells was examined using Mab T81. Fig. 3 shows immunofluorescent staining of cells infected with all three serotypes of MDV. Serotype 1

Table 1. Comparison of 21 blocks of amino acid similarity of RR between MDV, HSV, chicken and *E. coli*.

Block	RR subunit	MDV	HSV	Chicken	*E. coli*
1	RR1	SAL	SAL	RDF	RDM
2	RR1	SCYL	SCYL	SCFL	SCVL
3	RR1	GGIG	GGIG	GGIG	AGIG
4	RR1	EPWH	EPWH	EPWH	PMWH
5	RR1	LLF	LFF	LFM	LMY
6	RR1	S—EEFE	G—EEFE	G—EEFE	FADQEEFE
7	RR1	NLCTEI	NLCTEI	NLCTEI	NLCLEI
8	RR1	CSL	CNL	CNL	DEN
9	RR1	GIGVQGF	GIGMQGL	GIGVQGL	GIGVINF
10	RR1	NAQF	NAQF	NSLL	NSTL
11	RR1	MPT	MPT	MPT	MPS
12	RR1	KVT	KVT	RRV	KAS
13	RR1	KTG	KTAF	FTVW	IMQK
14	RR1	SQS	SQS	SQS	KVP
15	RR1	GLKTGMYY	GLKTGMYY	GLKTGMYY	GLKT-LYY
16	RR2	NRW	NRW	SFW	FFW
17	RR2	KDIHHYYF	KDILHYYV	TEARCFYG	PELETWVE
18	RR2	LIEGIFF	LIEGIFF	AVEGIFF	ALEAIRF
19	RR2	ISRDE	ISRDE	ISRDE	IARDE
20	RR2	SADR	SADR	VADR	ITNI
21	RR2	TNFFE	TNFFE	TNFFE	VAPQE

MDV strains used included GA (Fig. 3A), Md5 (Fig. 3B), or 648A (Fig. 3C), representing three different pathotypes of virulence, and vaccine strain CVI988 (Fig. 3D). Serotype-2, (SB-1; Fig. 3E) and serotype 3 (HVT; Fig. 3F) also reacted positively. The staining was found in the cytoplasm of the infected cells for all three serotypes (Fig. 3G, H) and not in uninfected DEF (Fig. 3I). In addition, our laboratory's collection of 51 strains of MDV, including 26 strains of serotype 1, 10 strains of serotypes 2, and 15 strains of serotype 3 viruses were positive with Mab T81, indicating that RR is a conserved protein in all three serotype of MDV.

Northern blot analysis. In order to analyze the transcription of the RR gene, total RNA from the GA strain of MDV-infected cells was isolated and probed with a 2.5-kb fragment containing the entire RR1 gene. As shown in Fig. 4, the probe detected a 4.4-kb and a 5-kb transcript. The 4.4-kb transcript was probably the UL39 and UL40 messenger RNA based on the size of the DNA as predicted from the nucleotide sequence and lack of polyadenylation (polyA) signal AATAAA downstream from UL39, but present at 40 nucleotides downstream from the stop codon of UL40. Therefore, there is only one coterminal transcript between these two open reading frames. The 5-kb transcript was predicted to include the UL38, UL39, and UL40 open reading frames since there is no PolyA signal downstream of UL38.

Immunoprecipitation and SDS-PAGE. To determine the molecular mass of the two RR subunits, MDV infected cells were subjected to immunoprecipitation with Mab T81. As seen in Fig. 5, two major proteins of approximately 90,000 and 40,000 daltons were detected on ^{35}S-methionine–labeled cells infected with MDV but not in uninfected DEF. The 90,000-dalton protein corresponds to the large-subunit RR1 and the 40,000 dalton protein corresponds to the small-subunit RR2. The RR1 of SB-1 (lane 3) appears to be slightly smaller than RR1 of GA (lane 2) and its RR2 appears to be slightly larger than that of GA (lane 2). For HVT (lane 4), the RR1 appears to be similar size as RR1 of GA (lane 2) but RR2 was not visible due to the fact that fewer infected DEF were used. We have previously shown that RR2 was in HVT-infected cells. Lane 1 represents DEF with Mab T81 as negative control.

Table 2. Mab T81 reacts with 51 strains of MDV belonging to three serotypes.

Serotype	Virus strain		
1	Md5	194	291
	Md7	238-1	296
	Md8	251	270
	Md9	284	272
	Md11	279C	279M
	Md11/75C	287L	293-12
	Md11/75C/R2	295	RPL39
	GA	JM	ALA-8
	648A	686	
2	SB-1	HN	6855
	280-5	281MI	287
	293-3	298B	301A
	301B		
3	FC125	AC16	AC18
	WTHV	HVTPA	239-1
	271B	280-3	281MD
	283	T3	T5
	T7	T8	T21

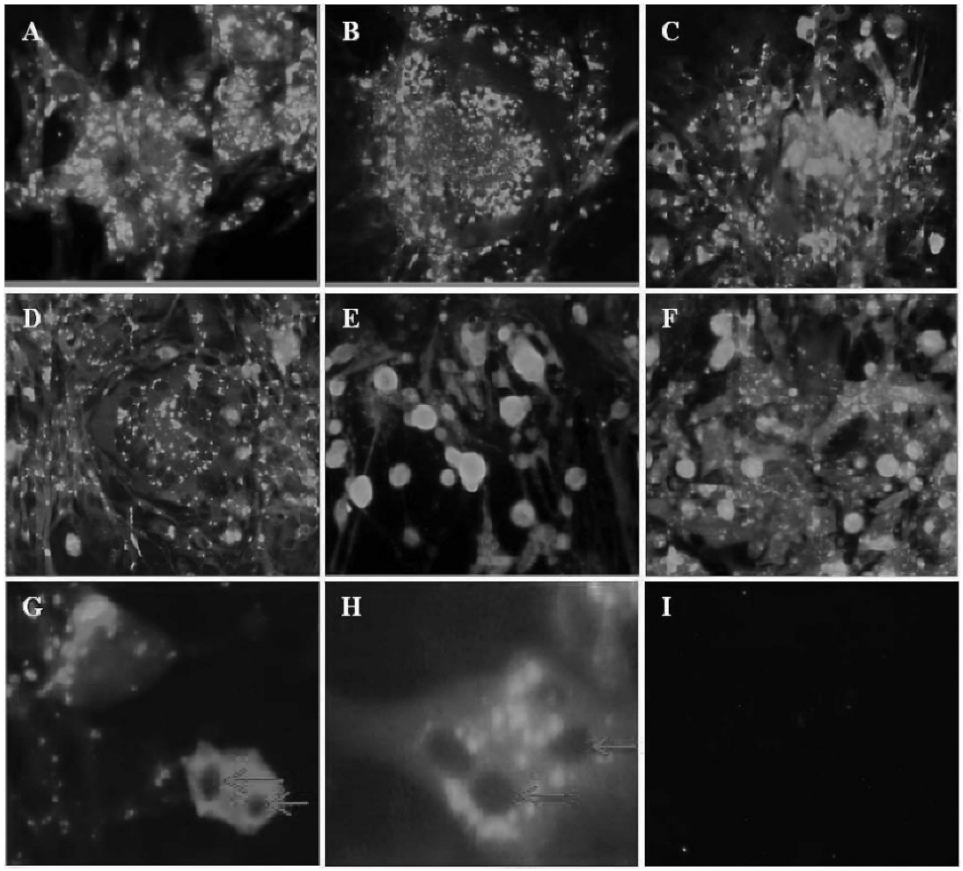


Fig. 3. Immunofluorescence for detection of RR expression in MDV-infected DEF cells with MAb T81. (A) GA-infected cell. (B) Md5-infected cell. (C) 648A-infected cell. (D) CVI988/Rispens-infected cell. (E) SB-1–infected cell. (F) HVT-infected cell. (G) Higher magnification of GA-infected cells with cytoplasmic staining, which is not present in the nuclei as indicated by arrows. (H) Higher magnification of Md5-infected cells with cytoplasmic staining. (I) Uninfected DEF as negative control.

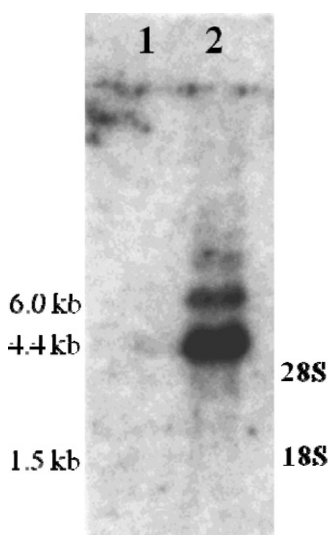


Fig. 4. Northern blot hybridization. Total RNA from DEF cells infected with GA-MDV was isolated at 48 hr postinfection. Twenty micrograms of RNA per lane was electrophoretically separated in an agarose gel and transferred to a nitrocellulose filter. The RNA filter was hybridized with a UL39-specific probe. Transcript size was determined by comparison to the BRL RNA ladder marker. Lane 1, uninfected DEF cells; lane 2, DEF cells infected with GA-MDV.

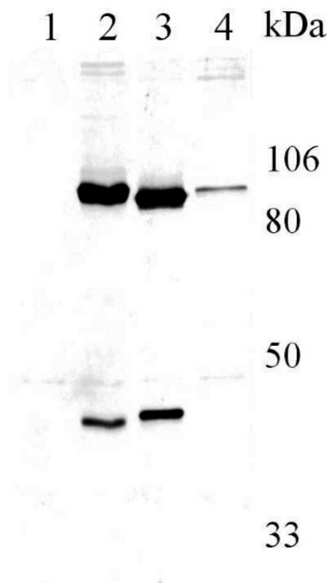
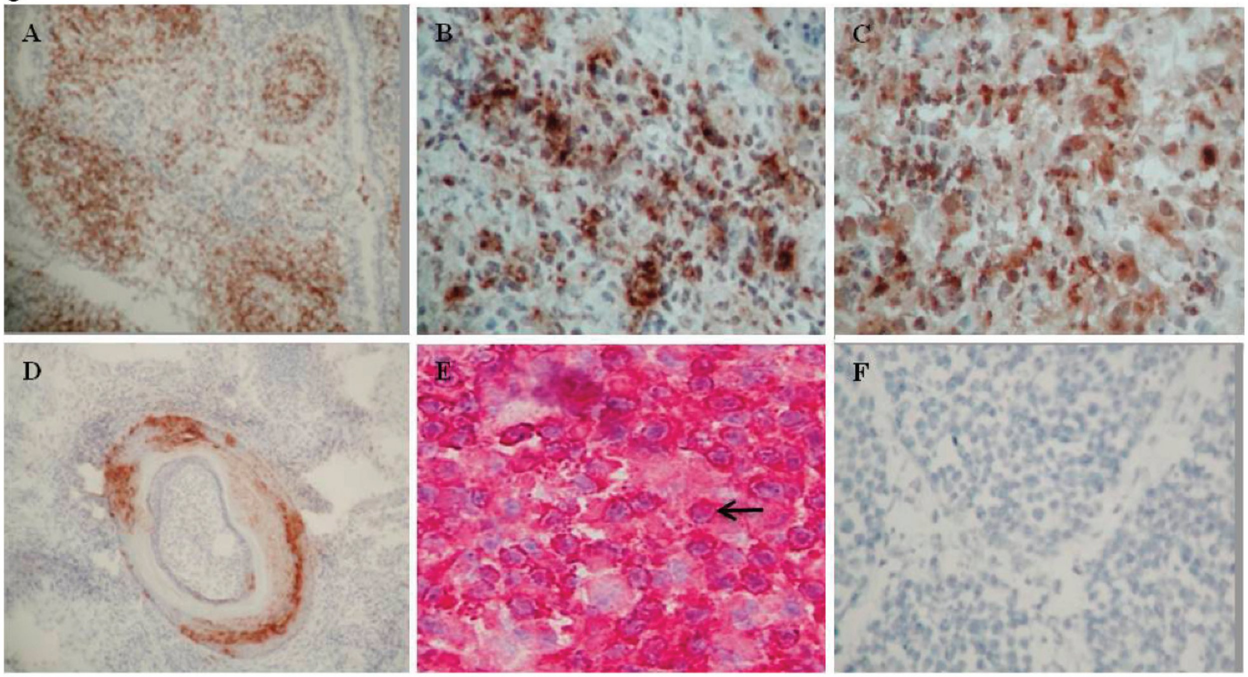


Fig. 5. Immunoprecipitation of RR with MAb T81 in three serotypes of MDV. DEF cells were labeled with ^{35}S-methionine 48 hr postinfection with GA-MDV. After 5 hr, the labeled cells were harvested and lysed in lysis buffer. Immunoprecipitation was carried out as described in Materials and Methods. The samples were analysis with 10% SDS-PAGE. Lane 1, DEF with Mab T81 as negative control; lane 2, GA with T81; lane 3, SB-1 with T81; lane 4, HVT with T81. Molecular weight markers are indicated as kDa.

6



7

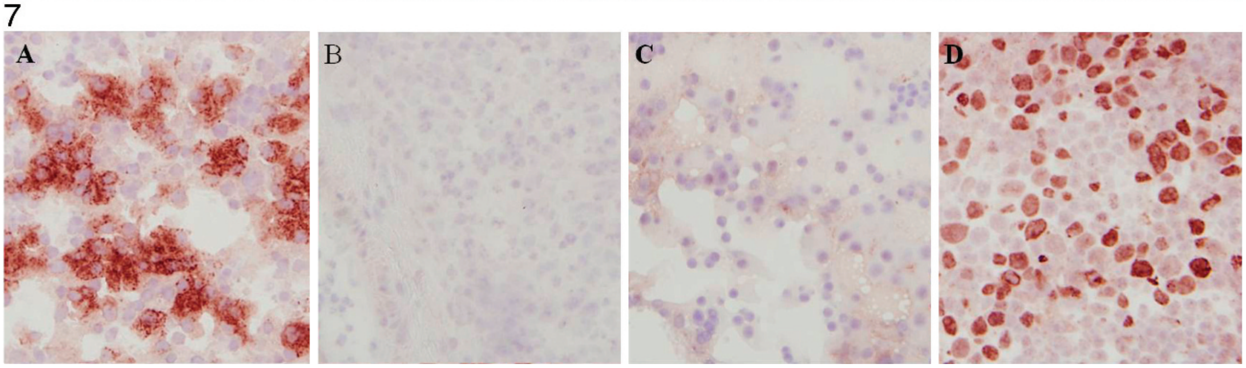


Fig. 6. Immunohistochemical analysis of lymphoid organs (bursa, spleen, and thymus) and FFE of GA-inoculated chickens at 6 days postinoculation. Frozen sections were stained with T81-specific Mab as described (12). (A) Bursa. (B) Spleen. (C) Thymus. (D) FFE (cross-section). (E) Higher magnification of bursa showing RR is expressed in the cytoplasm, not in the nucleus. (F) Thymus from uninfected chicken as negative control.

Fig. 7. Immunohistochemical studies of MDV tumors. This figure depicts the expression patterns of RR and the Meq oncogene in MDV-induced tumors. (A) RR is expressed in spleen tissue lacking any obvious tumors, whereas in (B), RR was not detected. (C) The Meq oncogene, a hallmark of MDV-induced tumors, was not detected in the same section of the spleen. In liver tissue with tumors, however, (D) meq was extensively expressed.

Identification of RR in MDV-infected chickens. The expression of RR during early cytolytic infection was studied in $15I_5 \times 7_1$ chickens inoculated with rMd5 (Fig. 6). RR was abundantly expressed in spleen (Fig. 6A), bursa (Fig. 6B), thymus (Fig. 6C), and feather follicle epithelium (FFE; Fig. 6D) of inoculated chickens and none was expressed in the uninoculated controls (Fig. 6F). To show that RR is expressed in the cytoplasm, a higher magnification of spleen tissue is shown in Fig. 6E. As shown, RR is expressed in the cytoplasm, not in the nucleus (see arrow). The expression of RR in FFE of two rMd5-infected chickens was studied at 14, 21, and 35 days after infection and RR was abundantly expressed at these time points. To study RR expression in GA-induced tumors, we stained serial sections from tumorous tissues with either Mab T81 for RR or specific antiserum for the Meq oncoprotein. Fig. 7 depicts the expression pattern of RR and Meq oncoprotein in MDV-induced tumors. RR is expressed in spleen tissue, lacking any obvious tumors (Fig. 7A). Meq oncogene, a hallmark of MDV-induced tumors, was not detected in the same section of the spleen (Fig. 7C). In liver tissue with tumors, however, Meq was extensively expressed (Fig. 7D) while RR was not detected (Fig. 7B).

DISCUSSION

We have previously determined that Mab T81 reacts with the protein expressed by UL39 of MDV serotype 1, RR (9). In this study we show that this Mab reacts with RR of 51 different strains of MDV belonging to all three serotypes in virus-infected cells. The expression of RR in cells infected with MDV but not in uninfected CEF cells suggests that the RR of MDV is different from that of chicken cells.

Northern blot analysis of RNA extracted from MDV-infected cells showed two large bands of 4.4 and 5 kb suggesting that the RR1 and RR2 transcripts have the same 3′ coterminal transcript as that of

HSV-1 (29). The RR proteins of MDV have molecular weights of approximately 90,000 (RR1) and 40,000 (RR2) daltons as shown by immunoprecipitation with Mab T81with all three serotypes of MDV. The RR2 band in HVT was not visible because fewer HVT-infected cells were used (Fig. 4). These two proteins were not found in uninfected DEF.

RR of herpesviruses is essential for viral pathogenesis and as such is a target for antiviral chemotherapy (7). The HSV-1 RR enzyme activity resides in a complex containing two molecules each of RR1 and RR2. HSV-encoded RR plays a key role in DNA synthesis and expression of this gene is required for growth in nondividing cells (13).

Numerous studies have shown that herpesvirus RR activity can be inhibited by a synthetic nonapeptide (YAGAVVNDL), corresponding to the nine carboxyl-terminal amino acids of the small subunit (6). This peptide is capable of interfering with normal subunit association that takes place through the carboxyl terminus of the small subunit (18). This inhibitory phenomenon observed with peptides appears not only to be universal, but also specific to the primary sequence of the enzyme (31). This binding of carboxyl-end peptides of RR1 and RR2 is of interest because of its possibilities as a basis for the design of species-specific antiproliferative drugs. This nonapeptide acts by competing for an RR2 binding site on RR1 and as a consequence disrupts the interaction between the two subunits (18).

The RR sequence of MDV resembles that of HSV-1 with significant sequence similarity distributed along the whole polypeptide except for the amine terminal (N-terminal) residues of HSV-1 related to protein kinase. This strongly suggests that the MDV RR may have the same overall three-dimensional structures and functions as that of HSV-1. From the MDV/HSV amino acid comparisons, two main features emerge, namely 1) the presence of an N-terminal region in HSV-1 RR1, which is absent from MDV, and 2) 21 discrete blocks of amino acid homology, some of which are shared with other RR polypeptides. The N-terminal region of HSV-1 RR1 also is absent from mouse and *E. coli* RR1 proteins. Homology between the MDV and HSV sequences is predominantly retained in blocks of conserved amino acids. The conservation between MDV and HSV-1 sequences is greater than that between HSV and EBV RR sequences (7). The presence of homologous sequences at different locations in MDV, HSV-1, and *E. coli* polypeptides is not likely to have arisen by chance and may have an evolutional importance.

All herpesvirus genomes encode a functional RR that is an essential enzyme for nucleotide metabolism and needed to convert ribonucleotides to deoxyribonucleotides, thus increasing the deoxynucleotide triphosphate pool for viral DNA synthesis in infected cells (20). As mentioned, RR of MDV is an essential enzyme for the conversion of ribonucleotides to deoxyribonucleotides and is expressed in lymphoid organs during the cytolytic infection of MDV. In MDV-induced tumors, however, our study confirms that RR is expressed abundantly in nontumorous tissues of MDV-infected chickens. Its expression, however, was not detected in tissues exhibiting virus-induced tumors. All these data taken together suggest that MDV genome encodes a functional RR that is essential for MDV replication in infected chickens but not involved in tumorigenesis.

REFERENCES

1. Afonso, C. L., E. R. Tulman, Z. Lu, L. Zsak, D. L. Rock, and G. F. Kutish. The genome of turkey herpesvirus. J. Virol. 75:971–978. 2001.

2. Bacon, L. D., H. D. Hunt, and H. H. Cheng. A review of the development of chicken lines to resolve genes determining resistance to diseases. Poult. Sci. 79:1082–1093. 2000.

3. Bulow, V. V., and P. M. Biggs. Differentiation between strains of Marek's disease virus and turkey herpesvirus by immunofluorescence assays. Avian Pathol. 4:133–146. 1975.

4. Cameron, J. M., I. McDougall, H. S. Marsden, V. G. Preston, D. M. Ryan, and J. H. Subak-Sharpe. Ribonucleotide reductase encoded by herpes simplex virus is a determinant of the pathogenicity of the virus in mice and a valid antiviral target. J. Gen. Virol. 69(Pt. 10):2607–2612. 1988.

5. Churchill, A. E., and P. M. Biggs. Agent of Marek's disease in tissue culture. Nature 215:528–530. 1967.

6. Cohen, E. A., P. Gaudreau, P. Brazeau, and Y. Langelier. Specific inhibition of herpesvirus ribonucleotide reductase by a nonapeptide derived from the carboxy terminus of subunit 2. Nature 321:441–443. 1986.

7. Conner, J., H. S. Marsden, and J. B. Clements. Ribonucleotide reductase of herpesviruses. Rev. Med. Virol. 4:25–34. 1994.

8. Conner, J., J. Murray, A. Cross, J. B. Clements, and H. S. Marsden. Intracellular localisation of herpes simplex virus type 1 ribonucleotide reductase subunits during infection of cultured cells. Virology 213:615–623. 1995.

9. Cui, Z., Y. Duan, and L. F. Lee. Identification and localization of the Marek's disease virus group-common antigen p79 gene. Chin. J. Virol. 9:66–72. 1993.

10. de Wind, N., A. Berns, A. Gielkens, and T. Kimman. Ribonucleotide reductase-deficient mutants of pseudorabies virus are avirulent for pigs and induce partial protective immunity. J. Gen. Virol. 74(Pt. 3):351–359. 1993.

11. Eidson, C. S., and S. C. Schmittle. Studies on acute Marek's disease. I. Characteristics of isolate GA in chickens. Avian Dis. 12:467–476. 1968.

12. Gimeno, I. M., R. L. Witter, H. D. Hunt, S. M. Reddy, L. F. Lee, and R. F. Silva. The pp38 gene of Marek's disease virus (MDV) is necessary for cytolytic infection of B cells and maintenance of the transformed state but not for cytolytic infection of the feather follicle epithelium and horizontal spread of MDV. J. Virol. 79:4545–4549. 2005.

13. Goldstein, D. J., and S. K. Weller. Herpes simplex virus type 1-induced ribonucleotide reductase activity is dispensable for virus growth and DNA synthesis: isolation and characterization of an ICP6 lacZ insertion mutant. J. Virol. 62:196–205. 1988.

14. Izumiya, Y., H. K. Jang, M. Ono, and T. Mikami. A complete genomic DNA sequence of Marek's disease virus type 2, strain HPRS24. Curr. Top. Microbiol. Immunol. 255:191–221. 2001.

15. Jordan, A., and P. Reichard. Ribonucleotide reductases. Annu. Rev. Biochem. 67:71–98. 1998.

16. Lee, L. F., X. Liu, and R. L. Witter. Monoclonal antibodies with specificity for three different serotypes of Marek's disease viruses in chickens. J. Immunol. 130:1003–1006. 1983.

17. Lee, L. F., P. Wu, D. Sui, D. Ren, J. Kamil, H. J. Kung, and R. L. Witter. The complete unique long sequence and the overall genomic organization of the GA strain of Marek's disease virus. Proc. Natl. Acad. Sci. U. S. A 97:6091–6096. 2000.

18. McClements, W., G. Yamanaka, V. Garsky, H. Perry, S. Bacchetti, R. Colonno, and R. B. Stein. Oligopeptides inhibit the ribonucleotide reductase of herpes simplex virus by causing subunit separation. Virology 162:270–273. 1988.

19. Nazerian, K., J. J. Solomon, R. L. Witter, and B. R. Burmester. Studies on the etiology of Marek's disease. II. Finding of a herpesvirus in cell culture. Proc. Soc. Exp. Biol. Med. 127:177–182. 1968.

20. Nikas, I., J. McLauchlan, A. J. Davison, W. R. Taylor, and J. B. Clements. Structural features of ribonucleotide reductase. Proteins 1:376–384. 1986.

21. Okazaki, W., H. G. Purchase, and B. R. Burmester. Protection against Marek's disease by vaccination with a herpesvirus of turkeys. Avian Dis. 14:413–429. 1970.

22. Preston, V. G., J. W. Palfreyman, and B. M. Dutia. Identification of a herpes simplex virus type 1 polypeptide which is a component of the virus-induced ribonucleotide reductase. J. Gen. Virol. 65(Pt. 9):1457–1466. 1984.

23. Reichard, P. Interactions between deoxyribonucleotide and DNA synthesis. Annu. Rev. Biochem. 57:349–374. 1988.

24. Rispens, B. H., H. van Vloten, N. Mastenbroek, H. J. Maas, and K. A. Schat. Control of Marek's disease in the Netherlands. I. Isolation of an

avirulent Marek's disease virus (strain CVI 988) and its use in laboratory vaccination trials. Avian Dis. 16:108–125. 1972.

25. Sambrook, J., E. F. Fritsch, and T. Maniatis. Molecular cloning: laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 1989.

26. Schat, K. A., and B. W. Calnek. Characterization of an apparently nononcogenic Marek's disease virus. J. Natl. Cancer Inst. 60:1075–1082. 1978.

27. Schumacher, D., B. K. Tischer, S. Trapp, and N. Osterrieder. The protein encoded by the US3 orthologue of Marek's disease virus is required for efficient de-envelopment of perinuclear virions and involved in actin stress fiber breakdown. J. Virol. 79:3987–3997. 2005.

28. Silva, R. F., and L. F. Lee. Monoclonal antibody-mediated immunoprecipitation of proteins from cells infected with Marek's disease virus or turkey herpesvirus. Virology 136:307–320. 1984.

29. Swain, M. A., and D. A. Galloway. Herpes simplex virus specifies two subunits of ribonucleotide reductase encoded by 3′-coterminal transcripts. J. Virol. 57:802–808. 1986.

30. Tulman, E. R., C. L. Afonso, Z. Lu, L. Zsak, D. L. Rock, and G. F. Kutish. The genome of a very virulent Marek's disease virus. J. Virol. 74:7980–7988. 2000.

31. Wierenga, R. K., M. C. H. De Maeyer, and W. G. J. Hol. Interaction of pyrophosphate moieties with α-helixes in dinucleotide binding proteins. Biochemistry 24:1346–1357. 1985.

32. Witter, R. L. Increased virulence of Marek's disease virus field isolates. Avian Dis. 41:149–163. 1997.

33. Witter, R. L., B. W. Calnek, C. Buscaglia, I. M. Gimeno, and K. A. Schat. Classification of Marek's disease viruses according to pathotype: philosophy and methodology. Avian Pathol. 34:75–90. 2005.

34. Witter, R. L., and L. F. Lee. Polyvalent Marek's disease vaccines: safety, efficacy and protective synergism in chickens with maternal antibodies. Avian Pathol. 13:75–92. 1984.

35. Witter, R. L., K. Nazerian, H. G. Purchase, and G. H. Burgoyne. Isolation from turkeys of a cell-associated herpesvirus antigenically related to Marek's disease virus. Am. J. Vet. Res. 31:525–538. 1970.